DISTINGUISHING PLASMODIUM FALCIPARUM TREATMENT FAILURES FROM RE-INFECTIONS BY USING POLYMERASE CHAIN REACTION GENOTYPING IN A HOLOENDEMIC AREA IN NORTHEASTERN TANZANIA

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Abstract. An in vivo drug sensitivity study was conducted in Magoda village in northeastern Tanzania to evaluate the usefulness of polymerase chain reaction (PCR)-based genotyping of Plasmodium falciparum parasites to distinguish between re-infection and treatment failure. The study tested P. falciparum susceptibility to a combination of sulfadoxine/pyrimethamine (Fansidar®; F. Hoffmann La Roche, Basel, Switzerland). Blood samples were collected before treatment and on days 7, 14, or 28 post-treatment in 51 asymptomatic children, of which 26 could not clear parasitemia within seven days post-treatment. Among the 25 children who had no detectable parasites on day 7, only five remained parasite negative up to day 28. Primary and recrudescent P. falciparum parasites were analyzed by PCR using family specific primers for merozoite surface protein-1 (MSP-1), MSP-2, and glutamate-rich protein (GLURP). All samples contained multiple P. falciparum infections. For all children with recrudescent P. falciparum, common alleles were detected in both the primary and recrudescent samples. However, in no child were the exact same alleles detected in both samples, indicating that probably at least some of the recrudescing parasites originated from new infections. The study demonstrates the general usefulness of PCR genotyping technique in distinguishing re-infections from true recrudescences following therapeutic drug treatment.

INTRODUCTION

Resistance of Plasmodium falciparum to antimalarial drugs is routinely classified into three grades: RI (low), RII (intermediate), and RIII (high).1 The RI type of response involves the reduction of parasitemia below the microscopic detection level for at least two weeks in the first seven days after treatment, followed by reappearance of parasites. In early recrudescence, the parasites are detectable before day 14 while in delayed recrudescence they appear between days 15 and 28 post-treatment. With RII type, there is a marked reduction of asexual parasitemia, but no parasite clearance, and with RIII there is slight or no marked reduction of asexual parasitemia.

In highly endemic areas with high inoculation rates, the parasitologic definition for RI grade response is faced with difficulties in ascertaining whether the recrudescence population is resistant and thus persisting from the initial infection or that such parasites are from a new infection.2,3 Reappearing parasites resulting from new infections may thus be falsely classified as treatment failures. Being able to distinguish between the two is very desirable to achieve proper evaluation of 1) antimalarial drug resistance studies, 2) malaria vaccine efficacy studies, and 3) effectiveness of current and novel antimalarial treatment regimens. However, there is currently no proven tool at hand to be used for this purpose, especially in holoendemic areas.

Interest has been indicated in exploiting the polymerase chain reaction (PCR) technique for such purposes. The few attempts to test this tool have been reported from areas with populations experiencing relatively lower entomologic inoculation rates (EIRs) such as Sudan,2 Gabon,2 and Papua New Guinea.3,5 The present study attempted to examine the usefulness of the PCR genotyping technique in an area of Tanzania with very high malaria endemicity (very high EIR) and with a high level of resistance to both sulfadoxine/pyrimethamine and chloroquine as previously shown by in vivo studies and dihydrofolate reductase (DHFR)6 or dihydropteroate synthetase (DHPH)5 gene point mutations.

MATERIALS AND METHODS

Study area. The study was carried out in Magoda village (5°11′S, 38°52′E) in the Muheza district in northeastern Tanzania. The area is holoendemic for malaria and has been described in detail elsewhere.3

Study population. The present drug efficacy trial was carried out during the long rainy season in April 1996 at the beginning of a malaria transmission peak. Children between one and nine years old were enrolled into the study. The children had to fulfill the following criteria: being of permanent residence in the village, being apparently healthy without fever, and having a P. falciparum monoinfection with >1,000 asexual parasites per microliter of blood. They also had to have no history of taking antimalarials for the past two weeks. Of the 144 screened children, 51 qualified for enrollment after an informed oral consent was obtained from their parents or guardians. The study was approved by the Ethical Committee of the National Institute for Medical Research, Tanzania, and the Central Scientific Ethical Committee, Denmark.

Examination, treatment, and follow-up. Thick and thin blood smears were prepared from a finger prick on day 0 immediately before drug administration. Blood samples were also blotted on filter paper (No. 1; Whatman, Hillsboro, OR) for later PCR analysis. The filter papers were dried at room temperature and placed into separate plastic bags, sealed, and stored at ~20°C. These samples, taken on day 0 were denoted as primary (P) samples.

A single dose of sulfadoxine/pyrimethamine (Fansidar®;
F. Hoffmann La Roche, Basel, Switzerland) was administered to each child at a target dosage of 1.25 mg of pyrimethamine/25 mg of sulfadoxine per kilogram of body weight. Following the treatment on day 0, the children were routinely monitored parasitologically on days 7, 14, or 28 after treatment. Beginning on day 7, follow-up was terminated upon confirming the presence of malaria parasites in the blood of a child. Thin and thick blood films, followed by a blood blot on filter paper, were made on each follow-up day as explained earlier. Follow-up filter paper blood blots from children positive for *P. falciparum* were denoted as recrudescent (R) samples. Only one recrudescent sample from each child was obtained and analyzed.

Thin films were fixed with methanol and both thick and thin films were stained with 10% Giemsa for 30 min. The number of asexual *P. falciparum* parasites per 200 leukocytes were counted under microscope using a high-power oil immersion objective, and the number of parasites was multiplied by 40 to calculate the number per microliter of blood. This assumed a mean leukocyte count of 8,000/µl. With low-density infestations, parasites were counted in 200 fields of standardized thick blood films. To calculate the number of parasites/µl, the 200 fields were estimated to represent a blood volume of 0.2 µl. A sample was declared negative after examining 200 thick film fields without observing any asexual parasites.

Children who cleared their parasites within the first seven days after treatment and whose blood films remained free from *P. falciparum* parasites until day 28 were considered to have had parasites that were fully sensitive (S) to pyrimethamine/sulfadoxine. Those who showed initial parasite clearance in the first week, followed by reappearance of parasites until day 28 were categorized as RI early or RI late mild responders, respectively. The other children who failed to clear their parasites during the first seven days, in some cases with slightly reduced or increased parasitemias, were considered to be non-responders in the RI/RII category.

**Extraction of DNA, PCR amplification, and product analysis.** Extraction and purification of DNA was performed using the Chelex method. Supernatants with DNA pellets were used immediately or stored at −70°C for later amplification of DNA. The polymorphic repetitive regions of block 2 of merozoite surface protein-1 (MSP-1), block 3 of MSP-2, glutamate-rich protein (GLURP) were amplified by a nested PCR. In a first reaction, oligonucleotide primer pairs corresponding to conserved sequences spanning the regions of the three genes were included in a single reaction. Using the product generated in the first reaction as template, six separate nested (second) reactions were performed using in each case a specific primer pair intended to determine the presence of allelic variants from the MAD20, K1, and RO33 families of MSP-1 block 2, the FC27 and the IC1 families of MSP-2 block 3, and the repetitive region of GLURP genes. The sequences of the primers for both outer and nested reactions for MSP-1 and MSP-2 genes have been fully described. The outer and nested GLURP primer sequences have been described elsewhere. Amplifications were performed using a GeneAmp® PCR System 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, CT). A nested PCR protocol design for standardized testing was used after minor modifications. In both outer and nested amplifications, reactions took place in a total volume of 20 µl containing 0.1 µl of 10% PCR buffer, 1.5 mM MgCl₂, 0.125 mM of the four dNTPs, and 0.4 units of AmpliTaq Gold Polymerase (Perkin Elmer, Emeryville, CA). Primers were used at a final concentration of 125 nM. One microliter of purified DNA corresponding to 1 µl of whole blood was used to initiate all the first reactions, and 1 µl of the product of that reaction was used for the six separate second (nested) reactions. The amplification profiles for both reactions had an initial denaturation period of 11 min at 94°C preceding the cycles: annealing for 120 sec at 58°C (or 60 sec at 61°C with nested reactions), extension for 120 sec at 72°C (or 60 sec at 72°C with nested reactions), and denaturation for 60 sec at 94°C (or 30 sec at 94°C with nested reactions). Twenty-five cycles were performed for the first reactions and 30 cycles were performed for all nested reactions. The last extension was carried out for 5 min at 72°C. After the addition of 5 µl of loading buffer to the final product, 10 µl were analyzed by agarose gel electrophoresis run on a 2.0% (or 2.5% for MSP-1) MetaPhor agarose gel (FMC BioProducts, Rockland, ME) in Tris-borate-EDTA buffer. For each child and marker type, a primary sample (P) was run alongside a recrudescent (R) sample. Following electrophoresis, DNA was visualized by ultraviolet transillumination after staining with ethidium bromide and fragment sizes were determined using standard size markers (100-basepair DNA Ladder or 100-basepair DNA Ladder Plus; MBI Fermentas, Fermentas Ltd., Vilnius, Lithuania).

**Data analysis and determination of recrudescence.** A pair of samples was analyzed by PCR from each individual: one before drug treatment (P) and one after parasites became patent (R) on either days 7, 14, or 28. A total of 26 pairs of samples picked randomly from those showing RI and RII/RIII type of responses were amplified by PCR. The presence of identical PCR products in both the P and R samples of an individual was defined as a (genuine) recrudescence of parasites. If the parasites in the P and R samples of an individual possessed exactly the same alleles of MSP-1, MSP-2, and GLURP, they were considered to represent a recrudescence of a single or multiple clone infection. If only a subset of the alleles found in sample P were found in the R sample, then the original P sample was considered to have had a mixture of both sensitive and resistant parasites.

On the other hand, if the parasites in the P and R samples were found to differ at all three loci, then parasites in the R sample were considered to clearly represent a new parasite population, most probably resulting from a re-infection. However, if the alleles were found to differ at only two loci, then the frequency of the common allele at the third locus was determined for the parasite population in all the individuals tested. If it was found to be high, then a re-infection was assumed to have taken place. However, if the frequency was found to be low, it was assumed that this was due to a recrudescence, since the chances of re-infection with parasites of the particular allele was considered low. Finally, if a sample was found to have identical alleles at two different loci (each one representing the major loci MSP-1, MSP-2, or GLURP), but differing at only one, then the parasites in sample R were considered recrudescent as a result of not responding to the drug.
The in vivo susceptibility study. Fifty-one children (age range = 1–9 years, mean age = 4.97 years) completed the follow-up. Only five children had complete clearance of parasites by day 7 and remained free of parasites by microscopy until day 28. Forty-one children showed an RI type of response, while five showed an RI/III type of response, indicating a drug failure rate (parasitologic) of 90%. The geometric mean parasite density was 3,744/μl before treatment (day 0) and decreased to 201/μl by day 7, as calculated only among those positive.

Genotyping by PCR. Twenty-six sets of samples were analyzed by PCR at three loci: MSP-1 block 2, MSP-2 block 3, and GLURP. All primary (P) samples were successfully amplified at all three loci. Four recrudescent (R) samples could not be amplified for at least one locus each, resulting in an amplification success of 93%, possibly due to low parasite densities. Twenty-two pairs of samples were successfully amplified at all target loci, and were thus included in the genotyping analysis. According to our classification criteria, all 22 children were confirmed to harbor parasites suspected to be resistant to pyrimethamine/sulfadoxine.

Mixed P. falciparum infections (6–28 alleles/sample) were detected in all children before and after treatment. The mean number of alleles per sample was estimated to be 9.6 before treatment and 6.5 after treatment. However, no child was found to harbor the same (sets of) alleles in both the P and R samples. Each primary and recrudescent sample possessed more than one allele on at least one of the three loci. Three children showed common alleles in the P and R samples at one locus only, and the particular common alleles had relatively high frequencies of occurrence in both the P and R parasite population for the whole cohort of children.

Two main patterns of recrudescences were observed. Type 1 involved the presence of a subset of alleles in the R sample that were previously present in the P sample together with an addition of new alleles; 18 children were in this category. Type 2 involved the presence of a subset of alleles in the R sample that were previously present in the P sample without addition of any new alleles: four children were in this category (Table 1). Table 2 shows the number of children with recrudescent parasites and those with both recrudescent and new parasites identified on days 7, 14, and 28 after treatment.

The distribution frequency of the prevalences of various allele types of each family or locus are shown in Figures 1 and 2. Allele prevalences are shown for both primary (P) and recrudescent (R) parasite populations. A range of 2–22 different sized PCR products were found for each of the allele family or locus observed.

### RESULTS

#### Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>No. children</th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI</td>
<td>18</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>RI/III</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>18</td>
<td>4</td>
</tr>
</tbody>
</table>

*Only one recrudescent sample showing the earliest presence of Plasmodium falciparum was analyzed.*

#### Table 2

<table>
<thead>
<tr>
<th>Day</th>
<th>No. examined</th>
<th>No. with drug failure* (%</th>
<th>No. with same recrudescent† alleles only</th>
<th>No. with recrudescent† plus new alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>26 (51)</td>
<td>7 (29)</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>13 (76)</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>28</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

*Refers to failure to clear parasites by that day, i.e., parasitologic versus clinical failure.† Only one recrudescent sample showing the earliest presence of Plasmodium falciparum was analyzed.*

#### DISCUSSION

Results from the in vivo sensitivity study suggest presence of a high level of resistance to pyrimethamine/sulfadoxine as previously indicated from studies in this area, although the parasitologic findings alone cannot exclude that some of the cases were due to re-infections. The PCR genotyping techniques have previously been shown to be particularly useful in differentiating recrudescences from re-infections in areas of comparatively low endemicity. Its use in areas of high endemicity is likely to provide strong evidence for distinction between re-infections and recrudescences. The only study done in an area with comparatively similar EIR using PCR and restriction fragment length polymorphism (PCR-RFLP) has been reported from Kilombero in southeastern Tanzania. However, the results from such analysis ought to be interpreted with some caution due to the high levels of genotype multiplicity and turnover rates prevailing in holoendemic areas.

Malaria parasites are haploid during their cycle in human hosts, and the three genes analyzed in this study exist only as single copies in the parasite genome. The presence of two or more alleles of any one gene (or gene family) in a single blood sample therefore denotes presence of a “mixed” infection with parasites of different genotypes.

Results from the PCR genotyping demonstrated that parasites re-appearing in the blood of children (R samples) suspected of harboring resistant P. falciparum parasites possessed the same alleles of MSP-1, MSP-2, and GLURP as those found in the primary (P sample) infections. Thus, similar allelic types were found in both primary and recrudescent infections in all 22 children. We therefore conclude that the recrudescent forms were derived from the resistant primary parasites and not from re-infections with new parasites.

The PCR analysis technique used in this study has a number of improvements compared with those used previously, which did not consider sequence variations in their analysis. We used three highly polymorphic loci, MSP-1, MSP-2 and GLURP, and have considered both sequence and size variations for MSP-1 and MSP-2 in our analysis. Both of these aspects improved the strength of our detection.
Estimating DNA fragment sizes from an agarose gel is not particularly accurate and has an estimated error of ≈ 10 basepairs. However, comparison of the P and R fragment pairs for each child was made by running the primary and recrudescent PCR products in tracks adjacent to each other on the same gel. This allowed even simple visual comparisons to be made, thus making the accurate estimate of the absolute fragment size less important in this aspect.
About 51% of the recrudescences occurred within seven days after treatment. This is in contrast to observations reported in Gabon. A possible explanation for this variation could be the difference in inoculation rates and parasite drug susceptibility levels between the two areas. This is reflected in the difference in proportions of multiple infections, which are comparatively more common (100%) in our study area than in Gabon (30%). The former study had shown that more than 70% of the samples had single clone infections in Gabon.
Two types of recrudescences were observed. Type 1 showed a subset of alleles from the P sample appearing in the R sample together with new alleles. This suggests that the child had a mixed infection possibly with a combination of both resistant and susceptible forms. Thus, the drug eliminated the susceptible parasites leaving behind the resistant ones. The additional new alleles may have appeared from a number of sources resulting from technical and biological factors. First, they may have been missed on day 0 due to being sequestered somewhere else, thus not being available in peripheral blood. Second, they may have been present but in comparatively low proportions such that they were out-competed for amplification by PCR. The third explanation could be that the parasites were still in the pre-erythrocytic stages and were not affected by the drug or were unavailable in peripheral blood at the time of sampling.

With type 2 recrudescence, a subset of alleles found in the P sample appeared again in the R sample without addition of new alleles. In such cases, it could be assumed that there was no sequestration on day 0 and the different genotypes existed in proportions well above the critical levels (1/10–1/100) at the time of sampling. This resulted into all parasites infecting the children to be adequately sampled in both the P and the R samples, followed by successful PCR amplification. The results further suggest that the drug simply eliminated the susceptible alleles leaving behind the resistant ones.

It appears that to make a reasonable conclusion on samples with mixed infections from holoendemic areas, it is important that one should have knowledge of the whole spectrum of parasite clones infecting an individual before and after drug treatment. The dynamics of parasite populations have been shown to be substantial, with different parasites detected on consecutive days in asymptomatic individuals with some 48-hr periodicity. Therefore, analysis of blood collected at different times should be considered, preferably even the day before and after drug administration as well as recurrences of parasites. Adopting this approach could prove useful in providing reliable parasite genotype baseline data. It remains to be seen whether integrating analysis of genes coding for resistance with genotyping could be of benefit in this kind of analysis. To date, such genes are not known for most antimalarials, with the exception of resistance to sulfadoxine/pyrimethamine, which has been associated with the DHFR* and DHPS* point mutations.

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